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FORM PTO-1390 (Modified) (REV 11-98)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER <b>201487/1060</b>
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR) <b>09/762867</b>
INTERNATIONAL APPLICATION NO. <b>PCT/JP99/03551</b>	INTERNATIONAL FILING DATE <b>01 July 1999</b>	PRIORITY DATE CLAIMED <b>18 August 1998</b>	
TITLE OF INVENTION <b>METHOD FOR ISOLATING SATELLITE SEQUENCES</b>			
APPLICANT(S) FOR DO/EO/US <b>TAKAHASHI, Hideaki; SEKINO, Masashi</b>			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:  1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371. 3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1). 4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371 (c) (2)) a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)). 7. <input type="checkbox"/> A copy of the International Search Report (PCT/ISA/210). 8. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3)) a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. 9. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 10. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)). 11. <input type="checkbox"/> A copy of the International Preliminary Examination Report (PCT/IPEA/409). 12. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).  <b>Items 13 to 20 below concern document(s) or information included:</b> 13. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 14. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 15. <input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment. 16. <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment. 17. <input type="checkbox"/> A substitute specification. 18. <input type="checkbox"/> A change of power of attorney and/or address letter. 19. <input checked="" type="checkbox"/> Certificate of Mailing by Express Mail 20. <input checked="" type="checkbox"/> Other items or information:  <b>UNSIGNED Combined Declaration and Power of attorney.</b> <b>Diskette containing sequence listing..</b> <b>Verified statement re sequence listing.</b>			

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.53) <b>09/762867</b>	INTERNATIONAL APPLICATION NO. <b>PCT/JP99/03551</b>	ATTORNEY'S DOCKET NUMBER <b>201487/1060</b>
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21. The following fees are submitted:.				<b>CALCULATIONS PTO USE ONLY</b>	
<b>BASIC NATIONAL FEE ( 37 CFR 1.492 (a) (1) - (5)) :</b>					
<input type="checkbox"/>	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO . . . . .	\$1,000.00			
<input checked="" type="checkbox"/>	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO . . . . .	\$860.00			
<input type="checkbox"/>	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO . . . . .	\$710.00			
<input type="checkbox"/>	International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) . . . . .	\$690.00			
<input type="checkbox"/>	International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) . . . . .	\$100.00			
<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				<b>\$860.00</b>	
Surcharge of \$130.00 for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492 (e)). <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30				<b>\$130.00</b>	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	9 - 20 =	0	x \$18.00	<b>\$0.00</b>	
Independent claims	1 - 3 =	0	x \$80.00	<b>\$0.00</b>	
Multiple Dependent Claims (check if applicable). <input type="checkbox"/>				<b>\$0.00</b>	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				<b>\$990.00</b>	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable). <input type="checkbox"/>				<b>\$0.00</b>	
<b>SUBTOTAL =</b>				<b>\$990.00</b>	
Processing fee of \$130.00 for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492 (f)). <input type="checkbox"/> 20 <input type="checkbox"/> 30 +				<b>\$0.00</b>	
<b>TOTAL NATIONAL FEE =</b>				<b>\$990.00</b>	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). <input type="checkbox"/>				<b>\$0.00</b>	
<b>TOTAL FEES ENCLOSED =</b>				<b>\$990.00</b>	
				Amount to be: refunded	\$
				charged	\$

- ☒ A check in the amount of **\$990.00** to cover the above fees is enclosed.
- ☐ Please charge my Deposit Account No. \_\_\_\_\_ in the amount of \_\_\_\_\_ to cover the above fees.  
A duplicate copy of this sheet is enclosed.
- ☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **14-1138** A duplicate copy of this sheet is enclosed.

**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

SEND ALL CORRESPONDENCE TO:

Michael L. Goldman  
Nixon Peabody LLP  
Clinton Square, P. O. Box 31051  
Rochester, New York 14603  
United States of America

SIGNATURE

Joseph M. Noto

NAME

32,163

REGISTRATION NUMBER

DATE

2/14/01



## DESCRIPTION

## METHOD FOR ISOLATING SATELLITE SEQUENCES

5 Technical Field

The present invention relates to a method for isolating satellite sequences. Satellite sequences, which are useful as population genetic markers, can be used as markers for genetic linkage analyses, etc. Therefore, efficient technique for isolating them is an important subject of research in the field of genome analysis.

## Background Art

It has been known that eukaryotic genomes contain repetitive sequences consisting of similar nucleotide sequence repeats. The first discovered repetitive sequence has a repeat unit consisting of satellite DNA, which is a long sequence ranging from several hundred to several thousand bases, and is called a satellite sequence (Bioscience, 27: 790-796, 1977). Repetitive sequences consisting of shorter nucleotide sequence units were later identified. They have been named, depending on the size of their repeat unit, microsatellite sequences (Am. J. Hum. Genet., 4: 397-401, 1989) when they have a repeat unit of 2 to 5 bases (Nucleic Acids Res. 9: 5931-5947, 1981) or minisatellite sequences (Nature, 314: 67-73, 1985) when they have a repeat unit of 10 to 64 bases (Nature, 295: 31-35, 1982). Microsatellite sequences are also called simple sequences (Nucleic Acids Res. 17: 6463-6471, 1989) or short tandem repeats (Am. J. Hum. Genet. 49: 746-756), etc.

Use of microsatellite sequences as markers was not reported when they were discovered. However, after their polymorphism was confirmed by polymerase chain reaction (PCR) (Am. J. Hum. Genet. 4: 397-401, 1989), they have attracted attention of researchers on their use as markers in a variety of areas. Specifically, their application to pedigree or lineage discrimination and individual identification of humans, animals, and plants is advanced. Because microsatellite sequences are scattered throughout a genome and abound in variation, they are good genetic markers. Furthermore, microsatellite DNA

polymorphism contains many polymorphic gene loci and a large number of alleles per gene locus. In addition, based on PCR, microsatellite DNA polymorphism analysis is easy to perform. The amplified products are easily detected as single or double bands by electrophoresis, which simplifies determination of individual types and facilitates the data processing. Therefore, microsatellite DNA markers have become widely used as the most effective markers for population genetics (J. Fish. Biol., 47: 29-55, 1995).

For microsatellite DNA polymorphism analysis, first, a variety of microsatellite DNA should be isolated from individual species to be analyzed. In addition, the microsatellite DNA regions should be amplified by PCR to detect the microsatellite polymorphism. Appropriate primers are needed for PCR. This means that, to perform microsatellite DNA polymorphism analysis, efficient methods for isolating microsatellite DNA from the species and for designing PCR primers capable of amplifying the microsatellite region are required.

The inventors have already reported a method that is expected to allow efficient isolation of microsatellites from poultry, for which the microsatellite polymorphism analysis is not advanced (Jpn. Poult. Sci., 33: 292-299, 1996). This method is an improvement on a conventional method of microsatellite sequence isolation that consists of a series of manipulations: fragmentation of a genome with restriction enzymes, insertion of the fragments into a vector, extension reaction with (TG)<sub>n</sub> primers, and then cloning (Proc. Natl. Acad. Sci. USA 89: 3419-3423, 1992). Namely, isolation of unknown microsatellite sequences was achieved by choosing a vector with high transformation efficiencies, by specifically digesting single-stranded DNA with mung bean nuclease, or by removing bacterial DNA and RNA with DNase I and RNase A. In chicken, it is considered difficult to efficiently isolate microsatellite sequences, because the number of microsatellite sequences is small. The above method could achieve, by calculation, six times more efficient isolation than the known method that was used in an attempt to isolate chicken microsatellite sequences (Poultry Science, 74: 1855-1874, 1995).

However, even by this method, it was impossible to prevent problematic clones from contaminating the microsatellite DNA clones

obtained. Namely, a high rate of duplicate clones was found among the clones obtained, suggesting bias in the constitution of clones as well as low efficiency.

5        Microsatellite sequences are required to be isolated according to each species. There are few species for which the isolation of microsatellite sequences is in progress, and it is still necessary to isolate microsatellite sequences from many species. However, there are a number of species-specific problems, for example, low frequency of microsatellite sequences in the chicken genome. Therefore, it is  
10        useful to provide a novel technique for isolating microsatellite sequences, also because it will enable selection of a variety of approaches.

#### Disclosure of the Invention

15        An objective of the present invention is to provide a method for isolating satellite sequences with high isolation efficiency. The present invention provides a highly efficient isolation method that any known technique cannot attain.

20        The present inventors presumed that one of the major causes that generate duplicate clones during isolation of satellite sequences by known techniques might come from the manipulation for fragmentation of the genomic DNA. In other words, the possibility that, when digested with restriction enzyme, the genomic library is biased due to the sequence-specific cleavage cannot be excluded.

25        Therefore, the inventors attempted to utilize a nucleotide sequence-independent cleavage method in which more arbitrary cleavage of the genomic DNA is expected. As a result, they found that cleavage of genomic DNA is possible with nucleotide sequence-independent DNA-digesting enzymes or by a physical action. However, since most  
30        of the physically cleaved genomic DNA fragments are not phosphorylated at their ends, they are not allowed to be efficiently incorporated into vectors using an enzyme. In addition, since physically cleaved fragments have an irregularly protruding strand at their cleavage site, they cannot be ligated. The inventors have overcome these  
35        problems by using several enzymatic treatments, and established a method to obtain more homogeneous genomic library. Furthermore, they

have confirmed that the library thus obtained enables efficient isolation of satellite sequences, thereby completing the present invention. As used herein, the term "satellite sequences" indicates, unless otherwise mentioned, any repeat sequences which include  
5 microsatellite sequences and minisatellite sequences. Also as used herein, "microsatellite sequences" and "minisatellite sequences" indicate repeat sequences consisting of repeat units of 2 to 5 bp and 10 to 64 bp, respectively. Thus, the present invention relates to the following methods for isolating satellite sequences.

10 (1) An isolation method for satellite sequences, wherein a genomic DNA is cleaved by a nucleotide sequence-independent method, the isolation method comprising:

a) obtaining randomly cleaved fragments of the genomic DNA and  
b) selecting, from the fragments obtained in a), fragments comprising  
15 the satellite sequences.

(2) The isolation method of (1), wherein the nucleotide sequence-independent method is a physical cleavage method or an enzymatic cleavage method.

(3) The isolation method of (2), wherein the physical cleavage method  
20 is sonication.

(4) The isolation method of (3), wherein the ends of the genomic DNA that have been fragmented by sonication are to be blunted.

(5) The isolation method of (4), wherein the ends are to be blunted with DNA polymerase having single strand-specific endonuclease  
25 activity and 3'→5' exonuclease activity.

(6) The isolation method of (2), wherein a nucleotide sequence-independent endonuclease is used in the enzymatic cleavage method.

(7) The isolation method of (6), wherein the nucleotide  
30 sequence-independent endonuclease is DNase I.

(8) The isolation method of (1), wherein the satellite sequences are microsatellite sequences.

(9) Use of satellite sequences isolated by the isolation method of any one of (1) to (8) as DNA markers.

35 In the present invention, it is critical to cleave a genomic DNA using a nucleotide sequence-independent cleavage method that gives

random fragments. Cleavage of a genomic DNA using a nucleotide sequence-independent cleavage method refers to cleaving DNA without dependence on structural characteristics (i.e., nucleotide sequences) of the DNA. Therefore, neither chemical reactions in which DNA is  
5 cleaved in a nucleotide sequence-dependent manner nor digestion with restriction enzymes constitutes the cleavage method of the present invention. The genomic DNA to which the treatment by these actions is to be applied can be prepared by known methods. Namely, cells are lysed enzymatically with protease, and are subjected to DNA extraction  
10 using an appropriate solvent.

For cleavage of a genomic DNA using a nucleotide sequence-independent cleavage method, any of cleavage methods, for example, those based on physical actions and those based on the action of DNA-digesting enzymes that do not recognize the nucleotide sequence,  
15 can be used. Physical actions include sonication and agitation. On the other hand, as DNA-digesting enzymes that do not recognize the nucleotide sequence, DNase I and the like are known. Any of these actions can be applied to the present invention. Above all, treatment by sonication is one of the most desirable manipulations with excellent  
20 reproducibility. By sonication, a large quantity of fragments with uniform lengths can be obtained in a short time. In contrast, partial digestion of a genomic DNA with DNase I requires strict setting of conditions for reproducibly obtaining fragments with uniform lengths. In addition, this method often requires troublesome manipulations.

25 As to the extent of cleavage of a genomic DNA, those skilled in the art can empirically set the conditions that will permit efficient production of fragments having the optimal size for the satellite sequence of interest. For example, if isolation of the microsatellite sequences is intended, such conditions that give a library of fragments  
30 of 300 to 900 bp are selected. Considering the operation of ligating the cleaved fragments into a vector, it is desirable to use conditions under which the fragments the majority of which have the size of 300 to 500 bp or 500 to 800 bp are generated. This is because, if fragments contain integral multiples of smaller fragments, such fragments are  
35 indistinguishable from the ligated products of more than one smaller fragment. For instance, when sonication at 20 kHz with amplitude of



10 is performed on ice, fragments of 300 to 500 bp can be efficiently obtained if the sonication of 1-minute duration is performed 1 to 5 times. Since a prolonged sonication may heat the sample and denature DNA, it is desirable to repeat the sonication with shorter time duration.

5       The genomic DNA cleaved by physical actions such as sonication tends to exhibit poor ligation efficiency, which prevents efficient incorporation of the DNA into vectors. For this reason, it is desirable to blunt the ends of the DNA fragments. In the present invention, any of the known blunting methods can be used: removal of the protruding  
10 ends with single strand-specific endonuclease, and further synthesis of the complementary strand at the 3' recessed ends with DNA polymerase to ensure the blunting. Specifically, mung bean nuclease and S1 nuclease can be used as the single strand-specific endonucleases. On the other hand, DNA polymerase having 3'→5' exonuclease activity  
15 can be used as the DNA polymerase. Such DNA polymerases include T4 DNA polymerase, pfu polymerase, and KOD polymerase. Mung bean nuclease, which is used in the Example, is a single strand-specific endonuclease and achieves blunting by digesting the protruding portion of the single strands of the DNA. In the Example, the action of T4 DNA polymerase  
20 is further utilized to ensure the blunting. T4 DNA polymerase digests the protrusion at the 3' ends by its strong 3'→5' exonuclease activity, and at the same time, it can synthesize complementary strands at the 3' recessed ends, thereby giving blunt ends. However, T4 DNA polymerase cannot blunt a recessed 3' end with a phosphate group.  
25 Therefore, it is desirable to use mung bean nuclease together. In such a case, treatment with mung bean nuclease cleaves gaps generated in the double-stranded DNA by sonication, as well as blunts the ends. As a result, fragments with lengths shorter than expected may be produced. In order to obtain a library free from shorter fragments, it is desirable  
30 to provide a step of isolating the fragments of expected size after treating with mung bean nuclease. Fragments of expected sizes can be isolated by agarose gel electrophoresis or by gel filtration. The blunt-ended genomic DNA fragments can be directly ligated into vectors. Alternatively, they can be phosphorylated at their 5' ends to improve  
35 the efficiency of ligation. T4 polynucleotide kinase can be used for phosphorylation.

The genomic DNA fragments with phosphorylated 5' ends can be self-ligated during the ligation into the vector. To prevent the self-ligation, the concentration of the fragments should be reduced, while excess concentration of the vector is employed so as to increase the opportunities for the fragments to be ligated into the vector. For the ligation reaction, T4 DNA ligase works in the ligation buffer and ligation efficiency can be further improved by carrying out the ligation in the presence of the restriction enzyme that have been used to cleave the vector. For example, when pCR-ScriptSK(+) is used as the vector, restriction enzyme SrfI can help keeping the vector in a linear state. Since SrfI does not act on the vector into which an insert is ligated, one can expect the ligation efficiency to be improved.

In the present invention, not only physical actions but also nucleotide sequence-independent DNA-digesting enzymes can be used for fragmentation of DNA. These enzymes include, for example, DNase I, etc. For the enzymatic treatment with DNase I, the conditions that allow the uniform action of the enzyme on the whole genome and that also allow production of the fragments with expected sizes should be empirically provided. For example, in order to maintain the enzymatic action in a uniform state, genomic DNA with highest purity should be used after nuclear proteins are removed. Also, the enzymatic reaction should be done as quickly as possible in order to avoid producing a large number of small fragments. In addition, conditions such as reaction time and temperature should be accurately controlled in order to maintain high reproducibility. After the enzymatic reaction is completed, the reaction is stopped by heating or other means. The nucleic acid component is recovered from the reaction, and, if necessary, particular fragments are extracted, followed by incorporation of the fragments into the vectors as described above. The fragments of the genomic DNA digested with the enzyme are blunt-ended and phosphorylated, which allows them to be directly used for ligation.

The vectors used in the present invention are not specially restricted. Specifically, known vectors such as pCR-ScriptSK(+), pBluescriptKS(+) (both are manufactured by Stratagene), and pUC18 can be used. After an appropriate host is transformed with the vector

including the insert and is grown, the DNA is recovered to make a genomic library. When pCR-ScriptSK(+) is used for transformation, it may be advantageous to use the host strains having excellent transformation efficiencies, such as *E. coli* (competent cell) XL1-Blue MRF', XL2-Blue MRF', and TG1 (all are manufactured by Stratagene).

Using the genomic library thus constructed, transformants containing the satellite sequences of interest can be cloned. Screening for the satellite sequences can be done based on the colony hybridization using a probe for a satellite sequence (Can. J. Fish. Biol. 51: 1959-1996, 1994) or on the primer extension (Proc. Natl. Acad. Sci. USA, 89: 3419-3423, 1992). In colony hybridization, colonies of the transformed cells as described above are transferred onto the filter, and hybridized with a probe having, for example, a repeat sequence of  $(GT)_n$ . The nucleotide sequence of oligonucleotide that constitutes the probe is properly provided based on the satellite sequence of interest. Positive colonies are separated to isolate the clones containing the satellite sequence.

In primer extension, on the other hand, the genomic library is recovered as single-stranded DNA. For example, if pCR-ScriptSK(+) is used as the vector, the transformed *E. coli* cells can be infected with helper phage VCS-M13 or the like to recover the inserts in a single-stranded form as recombinant phages. Since DNA packaged in the phage is protected against enzymatic action, RNA and DNA derived from *E. coli* can be enzymatically degraded when the DNA of interest is recovered, thereby achieving more efficient cloning.

To the single-stranded plasmid DNA isolated from the phage, a primer specific to the satellite sequence is annealed, and then, DNA polymerase is applied. For example, repetitive sequences of  $(dA-dT)_n$  are generally (0.3% of the entire genomic DNA) found in the mammalian microsatellite sequences. In humans, such combinations as  $(dC-dA/dT-dG)_n$  and  $(dC-dT/dA-dG)_n$  are also frequently observed. Based on such information, one can design primers that comprise a nucleotide sequence complementary to a satellite sequence and that anneal to the satellite sequence. It is advantageous to phosphorylate the 5' end of the primer beforehand. Vectors that contain a microsatellite sequence acquire the primer at this site and a double-stranded DNA

is synthesized, while those to which the primer does not anneal remain single-stranded. Ligation is performed after the synthesis of complementary strand with DNA polymerase to complete the double-stranded circular DNA, and then the single-stranded DNA is digested with mung bean nuclease or the like. Consequently, only the vectors containing the satellite sequences remain. These vectors can be recovered and used to transform cells, thereby cloning the vectors. After amplifying the cloned vectors containing the satellite sequences, one can recover the DNA and determine the sequences, thereby completing the isolation of the satellite sequences.

When a satellite sequence is isolated together with its flanking regions, primers for PCR can be designed. For designing the primers, it is convenient to utilize a commercially available package of software for sequence analysis. For example, in the Example below, a primer designing software Primer Premier (Premier Biosoft International) is used. The microsatellite sequences and minisatellite sequences isolated are useful as fingerprint markers for intraspecies analysis or as pedigree (or lineage) markers. If any satellite sequence obtained in the present invention exhibits polymorphism, such a nucleotide sequence can be used as an indicator for identification of individuals. Such a satellite sequence is called a fingerprint marker. Since satellite sequences used as fingerprint markers are inherited from the parents of an individual, analysis of the fingerprint markers conserved among multiple individuals indicates the probability that these individuals belong to a single pedigree. Such use of a satellite sequence is called a pedigree (or lineage) marker.

Furthermore, the satellite sequences isolated in the present invention can also be used as genetic linkage markers, in addition to the use as fingerprint markers and pedigree (or lineage) markers. In the present invention, a satellite marker utilized as an indicator represented by the uses exemplified above is called a DNA marker.

#### Brief Description of the Drawings

Figure 1 shows the coloring image of a dot-blot assay with a microsatellite sequence-specific probe ((CA)<sub>10</sub> oligonucleotide).

### Best Mode for Carrying out the Invention

The present invention is described more specifically in the following examples.

#### 5    (1) Preparation of DNA fragments from black abalone

Black abalone (*Haliotis discus discus*) DNA was isolated from the foot muscle according to the TNES-Urea method (Fisheries Sci., 62: 723-726, 1996). However, 4 M urea was used herein. The genomic DNA solution (20 µg/ml, 550 µl) was sonicated (20 kHz, Amplitude 10, 10    for one minute, five times) with cooling to fragment the DNA. The reaction mixture (52 µl) containing the substrate DNA (about 10 µg), 30 mM CH<sub>3</sub>COONa (pH 4.6), 50 mM NaCl, 1 mM (CH<sub>3</sub>COO)<sub>2</sub>Zn, 5% glycerol, and 60 units of mung bean nuclease (Toyobo) was incubated for 1 hour at 37°C to cleave the gap portions of the DNA fragments and to blunt 15    the protruding ends. By electrophoresis on a 1.2% agarose gel, the DNA fragments of 300 to 500 bp were recovered. The reaction mixture (100 µl) containing the recovered substrate DNA, 20 µM dNTP, 50 mM Tris/HCl (pH 8.5), 7 mM MgCl<sub>2</sub>, 15 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, and 10 units of T4 DNA polymerase (Toyobo) was incubated 20    for 1 hour at 37°C to blunt the protruding ends of the DNA fragments. Further, the reaction mixture (50 µl) containing the substrate DNA, 0.2 mM rATP, 50 mM Tris/HCl (pH 7.6), 10 mM MgCl<sub>2</sub>, 10 mM 2-mercaptoethanol, and 10 units of T4 polynucleotide kinase (Toyobo) was incubated for 1 hour at 37°C to phosphorylate the 5' ends of the DNA fragments.

25

#### (2) Ligation of genomic DNA fragments into plasmid vectors

The reaction mixture (10 µl) containing pCR-Script SK(+) vector (Stratagene) (about 1 µg), 25 mM Tris/Acetate (pH 7.6), 100 mM KOAc, 10 mM MgOAc, 0.5 mM 2-mercaptoethanol, 10 µg/ml BSA, and 10 units 30    of restriction enzyme SrfI (Stratagene) was incubated for 1 hour or more at 37°C, to cleave the vector at the SrfI site. The reaction mixture (50 µl) containing the vector solution prepared, the substrate DNA (about 3 µg), 10 units of SrfI, 0.5 mM rATP, 66 mM Tris/HCl (pH 7.6), 6.6 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, and 20 units of T4 DNA ligase 35    (Toyobo) was incubated overnight at 24°C to ligate the DNA fragments into the vector. After inactivating the DNA ligase by heating at 65°C

for 15 minutes, 10 units of SrfI was further added. The reaction mixture was incubated for 1 hour at 37°C to digest the self-ligated vector.

### (3) Preparation of single-stranded DNA

5 To each of five culturing tubes, 100 µl of XL2-Blue MRF' ultracompetent cells (Epicurian coli ultracompetent cells; Stratagene) was added, and then, the ligated vector (about 200 ng) was added, followed by transformation according to the attached instruction manual. After 900 µl of the NZY medium (Maniatis, 10 Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York, 1989) was added to each of the tubes, the cells were cultured for one hour at 37°C with shaking. Subsequently, ampicillin was added to the final concentration of 50 µg/ml. The cells were cultured for another one hour at 37°C with shaking to select 15 the transformed *E. coli*. To each of the tubes, about 10<sup>10</sup> pfu of VCS-M13 helper phage was added. The cells were allowed to stand for 20 minutes at 37°C, followed by addition of kanamycin at the final concentration of 70 µg/ml. The cells were cultured for 1 hour at 37°C with shaking to select the *E. coli* infected with the helper phage. All the cultures 20 were put together and added to 100 ml of the Terrific medium (Maniatis, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York, 1989) containing ampicillin and kanamycin. The cells were cultured for 14 hours at 37°C with shaking. The Terrific medium was centrifuged at 14,000 rpm for 10 minutes at 0°C, and the 25 supernatant was recovered and filtered twice through a 45 µm filter. After precipitated twice with PEG according to the standard method (Muramatsu, Lab Manual of Genetic Engineering, 3rd ed., Maruzen, Tokyo, 1996, pp.51-55), the DNA and RNA derived from *E. coli* were digested according to a known method (Jpn. Poult. Sci. 33: 292-299, 1996). 30 Subsequently, PEG precipitation, phenol extraction, and ethanol precipitation were performed to purify the single-stranded DNA.

### (4) Selection of (CA)<sub>n</sub>-positive plasmids

Primer extension was performed using the (CA)<sub>12</sub> oligonucleotide 35 to select the plasmid DNA into which DNA fragments with (TG/CA) repeats had been inserted. The solution (98 µl) containing the single-stranded

DNA (about 3  $\mu$ g), 0.2 mM dNTP, 20 mM Tris/HCl (pH 8.8), 10 mM KCl, 10 mM  $(\text{NH}_4)_2\text{SO}_4$ , 2 mM  $\text{MgSO}_4$ , 100  $\mu$ g/ml BSA, 0.1% Triton X-100, and 100 pmol  $(\text{CA})_{12}$  oligonucleotide was pre-heated at 72°C for 10 minutes. Five units of Pfu DNA polymerase (Stratagene) was added to the solution, which was then overlaid with mineral oil and incubated for 30 minutes at 72°C. Subsequently, the generated double-stranded DNA was recovered by phenol extraction and ethanol precipitation and was dissolved in 10  $\mu$ l of sterilized water. After circularizing the double-stranded DNA by using the ligation kit (Ligation high, Toyobo), the DNA ligase was inactivated by heat treatment (65°C for 15 minutes). The reaction mixture (100  $\mu$ l) containing this solution, 30 mM  $\text{CH}_3\text{COONa}$  (pH 4.6), 50 mM NaCl, 1 mM  $(\text{CH}_3\text{COO})_2\text{Zn}$ , 5% glycerol, and 30 units of mung bean nuclease (Toyobo) was incubated for 2 hours or more at 37°C to digest the single-stranded DNA which had not been primer-extended. DNA was recovered by phenol/chloroform extraction and ethanol precipitation, and was dissolved in 60  $\mu$ l of TE buffer.

With the recovered DNA (equivalent to 100  $\mu$ g of the single-stranded DNA), the XL2-Blue MRF' Ultracompetent cells were transformed. The transformed cells were spread onto a 2xYT agar medium containing 50  $\mu$ g/ml ampicillin (Maniatis, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York, 1989), and cultured overnight at 37°C. Single colonies on the culture plate were picked up at random, and cultured overnight at 37°C with shaking in 2 ml of 2xYT medium (Maniatis, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York, 1989) containing ampicillin at the final concentration of 50  $\mu$ g/ml. The plasmid was extracted by the alkaline method according to the standard procedure (Muramatsu, Lab Manual of Genetic Engineering, 3rd ed., Maruzen, Tokyo, 1996, pp.51-55), and dissolved in 50  $\mu$ l of TE buffer.

The purified plasmid DNA (equivalent to 4 ng of the single-stranded DNA) was alkaline-denatured with 0.5 N NaOH and blotted onto a nylon membrane (No. 1209299, Boehringer Mannheim). The membrane was baked at 120°C for 30 minutes. Using  $(\text{CA})_{10}$  oligonucleotide digoxigenin-labeled with the DIG oligonucleotide labeling kit (DIG Oligonucleotide Tailing Kit, Boehringer Mannheim) as a probe, and

using the DIG nucleic acid detection kit (DIG Nucleic Acid Detection Kit, Boehringer Mannheim), (CA)<sub>n</sub>-positive plasmids were detected according to the manufacturer's instruction. Part of the results is shown in Figure 1.

5

#### (5) Cycle sequencing

For the (CA)<sub>n</sub>-positive clones, nucleotide sequences were determined using cycle sequencing method. The KS primer labeled with Cy5 at its 5' end, the Reverse primer, and the sequencing kit (ThermoSequence fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP; Amersham) were used for the sequencing reaction. The fluorescent DNA sequencer (ALFexpress, Pharmacia) was used for sequencing. Based on the nucleotide sequence data thus obtained, optimal primers were designed for the region encompassing the CA repeats using software for primer design (Primer Premier; Premier Biosoft International). The nucleotide sequences of the microsatellite sequences isolated from the black abalone are shown below. Repetitive units are shown in the parentheses so that they are easily recognized. The actual sequences are as shown in the sequence listing (the numbers correspond to the SEQ ID NOs). For the regions 1 to 24, PCR primers can be designed, and polymorphism was identified for the nine regions (1, 3, 8, 10, 13, 15, 16, 19, and 24).

1. (CA)<sub>41</sub>
2. (GACT)<sub>2</sub>(CTCA)<sub>7</sub>(CA)<sub>2</sub>CT(CA)<sub>9</sub>
- 25 3. C<sub>5</sub>CAC<sub>2</sub>(CA)<sub>12</sub>TA(CA)<sub>8</sub>
4. (CA)<sub>7</sub>
5. (CA)<sub>16</sub>
6. (GA)<sub>2</sub>CAGA(CA)<sub>5</sub>
7. CA<sub>3</sub>GA<sub>2</sub>C<sub>3</sub>A<sub>3</sub>(CA)<sub>5</sub>
- 30 8. (CGCA)<sub>9</sub>TGCAC<sub>2</sub>(CA)<sub>2</sub>
9. (CT)<sub>3</sub>(CA<sub>2</sub>)<sub>3</sub>(CA)<sub>5</sub>
10. (CA)<sub>25</sub>
11. CACT(CA)<sub>16</sub>TACA
12. CA<sub>3</sub>(CA)<sub>2</sub>T(CA)<sub>4</sub>
- 35 13. (CA)<sub>30</sub>
14. CA<sub>2</sub>GCA<sub>2</sub>C(CA)<sub>25</sub>



15.  $(CA)_2CT(CA)_{13}(CGCA)_{11}(CA)_6$
16.  $(CA)_8(CG)_4$
17.  $(CA)_6(CG)_4$
18.  $(CA)_5$
- 5 19.  $(CA)_{26}$
20. TACATA $(CA)_{12}$
21.  $(CA)_2CA_3(CA)_6$
22.  $(CA)_2AC(CA)_3AC(CAC)_2(CA)_5$
23.  $(CA)_8(TGCA)_2$
- 10 24.  $(CA)_{34}$
25.  $(CA)_7(CGCA)_2CGA_2(CGCA)_2A_2(CA)_2(CG)_2$
26.  $(CA)_8$
27.  $CAC_8(CA)_9C_4$
28.  $(CA)_6(GA)_2$
- 15 29.  $(CA)_{26}$
30.  $(CA)_{25}$
31. CAG $(CA)_5$ TACA
32.  $(CA_3)_3(CA)_4CA_3(CA)_{12}G_2CA(CG_2)_3$

About 85% (41 clones) of the  $(TG/CA)_n$ -concentrated library constructed based on the sequence-independent cleavage method using sonication was found to be  $(CA)_n$ -positive clones (Figure 1). Out of them, 32  $(CA)_n$ -positive clones were selected at random and sequenced. As a result, the PCR primers can be designed for 24 clones (72%). For the rest of the clones (9 clones), it was impossible to design the primers because some repeat regions were adjacent to the ligation site on the vector, because some regions were ligated into the vector in the middle of the repeat, or because some repeats were scattered throughout the DNA fragment.

### 30 Industrial Applicability

The present invention makes it possible to efficiently isolate satellite sequences such as microsatellite sequences. Since the present invention utilizes a sequence-independent cleavage method for fragmentation of a genomic DNA, it is not influenced by the nucleotide sequence, and enables producing unbiased libraries. Satellite sequences isolated from the unbiased library contain reduced

redundancy. Thus, the present invention enables efficiently isolating satellite sequences.

For instance, the present inventors have succeeded in obtaining a (TG/CA)<sub>n</sub>-concentrated library comprising 32 clones by a single manipulation from black abalone from which no microsatellite sequences has been isolated previously. The clones obtained from this library are free from redundancy. It can be concluded that, compared with the previous method that produced many duplicated clones for chicken, the present method has increased the efficiency of the isolation. The isolated clones can serve for designing PCR primers as well as show reduced sequence redundancy, which is another striking effect of the present invention. For instance, calculated from the result of the example, at least 60% ( $0.85 \times 24/32$ ) of the clones cultured on the plate can be used to design PCR primers. These results reveal that the methods of the present invention for isolating microsatellite sequences are excellent methods that can be widely applied.

Furthermore, the satellite sequences isolated by the methods of the present invention can be utilized as DNA markers, such as fingerprint markers, pedigree (or lineage) markers, and genetic linkage markers.

## CLAIMS

1. An isolation method for satellite sequences, wherein a genomic DNA is cleaved by a nucleotide sequence-independent method, the isolation method comprising:
  - a) obtaining randomly cleaved fragments of the genomic DNA and
  - b) selecting, from the fragments obtained in a), fragments comprising the satellite sequences
2. The isolation method of claim 1, wherein the nucleotide sequence-independent method is a physical cleavage method or an enzymatic cleavage method.
3. The isolation method of claim 2, wherein the physical cleavage method is sonication.
4. The isolation method of claim 3, wherein the ends of the genomic DNA that have been fragmented by sonication are to be blunted.
5. The isolation method of claim 4, wherein the ends are to be blunted with DNA polymerase having single strand-specific endonuclease activity and 3' → 5' exonuclease activity.
6. The isolation method of claim 2, wherein a nucleotide sequence-independent endonuclease is used in the enzymatic cleavage method.
7. The isolation method of claim 6, wherein the nucleotide sequence-independent endonuclease is DNase I.
8. The isolation method of claim 1, wherein the satellite sequences are microsatellite sequences.
9. Use of satellite sequences isolated by the isolation method of any one of claims 1 to 8 as DNA markers.

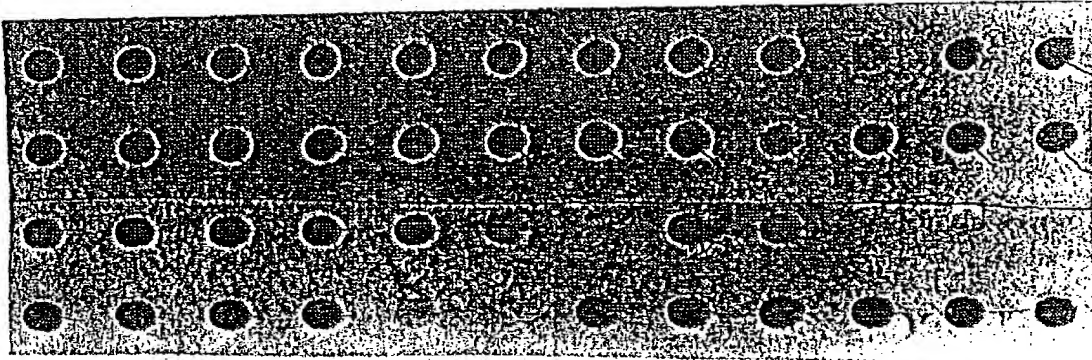
## ABSTRACT

A highly homogeneous library can be obtained by cleaving a genomic DNA by a sequence-independent cleavage method, such as sonication.

- 5 By selecting satellite sequences from such a library, efficiency of isolation is improved. Thus, an efficient method of isolating microsatellite sequences, which are useful as DNA markers, is provided.

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Figure 1



I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201 <i>Ridecker Sakahashi</i>	SIGNATURE OF INVENTOR 202 <i>Masashi Sekine</i>	SIGNATURE OF INVENTOR 203
DATE July 19, 2001	DATE July 24, 2001	DATE

COMBINED DECLARATION FOR PATENT  
APPLICATION AND POWER OF ATTORNEY  
(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER  
201487/1060

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**METHOD FOR ISOLATING SATELLITE SEQUENCES**

the specification of which (check only one item below):

- ☐ is attached hereto.
- ☐ was filed as U.S. Patent Application Serial No. \_\_\_\_\_ on \_\_\_\_\_ and was amended on \_\_\_\_\_ (if applicable).
- ☒ was filed as PCT International Application Number PCT/JP99/03551 on July 1, 1999 and was assigned U.S. Serial No. 09/762,867.

I hereby state that I have reviewed and understand the contents of the above-identified specifications, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (IF PCT, indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
PCT	PCT/JP99/03551	1 July 1999	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
Japan	10/232153	18 August 1998	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
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COMBINED DECLARATION FOR PATENT  
APPLICATION AND POWER OF ATTORNEY (Continued)  
(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER  
201487/1060

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT International filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

U.S. APPLICATIONS			STATUS (Check One)		
U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED	
PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NO.	PCT FILING DATE	U.S. SERIAL NUMBERS ASSIGNED (if any)			

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. Michael L. Goldman, Registration No. 30,727; Joseph M. Noto, Registration No. 32,163; Grant E. Pollack, Registration No. 34,097; Ann R. Pokalsky, Registration No. 34,697; Gunnar G. Leinberg, Registration No. 35,584; Edwin V. Merkel, Registration No. 40,087; Georgia Evans, Registration No. 44,597; Alice Y. Choi, Registration No. 45,758

Send Correspondence to:

Michael L. Goldman, Esq.  
NIXON PEABODY LLP  
Clinton Square, P.O. Box 31051  
Rochester, New York 14603

Direct telephone calls to:  
Michael L. Goldman  
(716) 263-13040

1-00	FULL NAME OF INVENTOR	FAMILY NAME <u>Takahashi</u>	FIRST GIVEN NAME <u>Hideaki</u>	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY <u>Tsukuba-shi</u>	STATE/FOREIGN COUNTRY <u>Ibaraki</u>	COUNTRY OF CITIZENSHIP <u>Japan</u>
	POST OFFICE ADDRESS	P.O. ADDRESS <u>c/o National Institute of Agrobiological Sciences, 2-1-2, Kannondai</u>	CITY <u>Tsukuba-shi</u>	STATE & ZIP CODE/COUNTRY <u>Ibaraki 305-8602 Japan</u>
2-01	FULL NAME OF INVENTOR	FAMILY NAME <u>Sekino</u>	FIRST GIVEN NAME <u>Masashi</u>	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY <u>Shiogama-shi</u>	STATE/FOREIGN COUNTRY <u>Miyagi</u>	COUNTRY OF CITIZENSHIP <u>Japan</u>
	POST OFFICE ADDRESS	P.O. ADDRESS <u>c/o Tohoku National Fisheries Research Institute 3-27-5, Shinhamma-cho</u>	CITY <u>Shiogama-shi</u>	STATE & ZIP CODE/COUNTRY <u>Miyagi 985-0001 Japan</u>
2-03	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE/FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
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Sekino, Masashi

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<140> 09/762,867

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24

&lt;210&gt; 24

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&lt;212&gt; DNA

&lt;213&gt; Haliotis discus discus

9/11

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&lt;213&gt; Haliotis discus discus



10/11

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32

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52

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61